METHODS OF ISOLATION AND CULTIVATION OF ANAEROBIC BACTERIA

STUDIES IN BACTERIAL METABOLISM. XLIII

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The isolation of anaerobic bacilli in pure culture and their subsequent development in a state of unquestioned freedom from contamination presents one of the more difficult and complex problems of bacteriologic procedure. Prior to the recognition of the prominent part played by anaerobic bacilli as incitants of gas gangrene and similar conditions associated with wounds of warfare, the study of these organisms as a group was much neglected and the technic of anaerobiotic procedure was incompletely developed. For this reason the most striking characteristic of the anaerobic group has been overlooked, namely, the ability of certain members to grow side by side as "pure mixed cultures" for long continued serial transfers on artificial mediums. It must be admitted, however, that the recognition of contaminants in supposedly pure strains of anaerobic bacilli is much more difficult of accomplishment than would appear at first sight. This is due in part to the fact that available descriptions of the anaerobic organisms are for the most part incomplete or inaccurate, both culturally and with reference to morphology and sporulation. Furthermore, the widespread distribution of members of this group in nature provides abundant opportunity for the symbiotic association of unlike strains whose combined activities are mutually advantageous. It is not improbable that the effect of reduced oxygen tension, which is a requirement for anaerobic growth, may in itself have been a potent environmental factor in promoting symbiotic relationships among the members of the group of anaerobic bacilli, although aerobic organisms, such as Micrococcus ovalis, are not uncommon contaminants of anaerobic cultures.

Theories and even controversies have arisen from studies on cultures of anaerobic bacilli containing unrecognized and therefore unsus-

Received for publication May 6, 1921.

pected contaminants. A striking, although not vital, instance is that vigorous discussion which has centered around the conception of "putrefaction," as a bacterial process. A small group of investigators

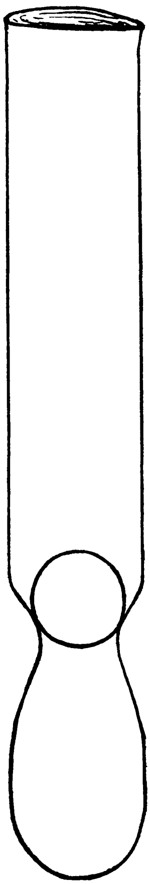

Fig. 1.—Test tube used in experiment performed according to method of Hall.

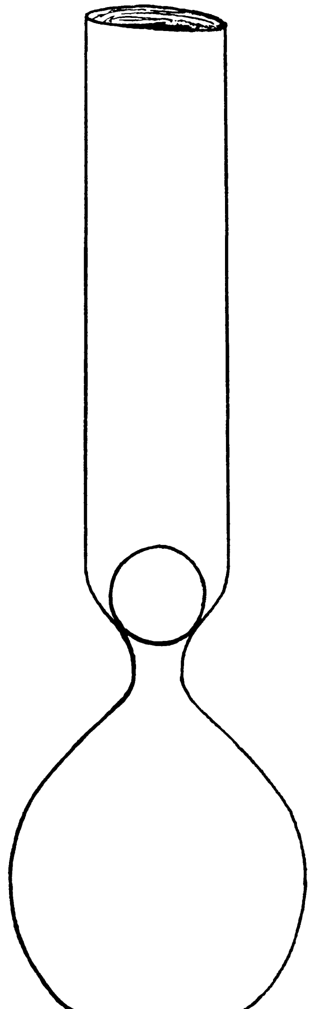

Fig. 2.—Modification of test tube in fig. 1.

have maintained that putrefaction is incited only by anaerobic bacteria, of which Bacillus putrificus,[1] the bacillus of malignant edema, and of

[1] Bienstock: Ztschr. f. klin. Med., 1884, 3, p. 1; Arch. f. Hyg., 1899, 36, p. 335; 1901, 39, p. 335. Rettger: Jour. Biol. Chem., 1906-1907, 2, p. 71; 1908, 4, p. 15. Rettger and Newell, ibid., 1912, 13, p. 341.

symptomatic anthrax are regarded as the principal, if not the sole, etiologic types. Putrefaction has been defined by these observers as a decomposition of protein or protein derivatives by these organisms, resulting in the formation of certain foul smelling products. In this respect the process seems to be somewhat similar chemically to the process called "Fäulnis" by the Germans. It is now believed that the bacillus of malignant edema (Vibrion septique), the bacillus of symptomatic anthrax, and even Bacillus putrificus,[2] freed from contaminating organisms, are distinguished by their feeble action on protein derivatives. Bacillus tetani and Bacillus botulinus were also formerly regarded as strong proteolytic organisms, but subsequent investigations have shown that powerfully toxicogenic strains, which are practically devoid of proteolytic powers, may be isolated from supposedly pure cultures of these bacteria.

A striking instance of the effect which unrecognized anaerobic symbiosis may have on current bacteriologic thought is that remarkable "dimorphism" of the bacillus of symptomatic anthrax, studied by Grassberger and Schattenfroh, and summarized in Sittler's monograph on intestinal bacteria.[3] The bacillus of symptomatic anthrax, according to these investigations, is said to undergo the following series of transformations as the environmental conditions to which the organism is subjected are changed.[4]

BACILLUS OF SYMPTOMATIC ANTHRAX

Terminal spore type		Clostridial type (Gas phlegmon bacillus)
↕	"denaturable" nonmotile, nonsporogenic butyric acid bacillus	↕
related to		related to
↕		↕
Bacillus putrificus (Putrefactive phase develops in absence of utilizable carbohydrate)		Anaerobic, motile, butyric acid bacillus (fermentative phase develops in presence of utilizable carbohydrate)

It is now believed that this apparent transmigration of vital energy through a series of nodes of relative cultural stability was due to the

[2] Medical Research Committee: Report on the Anaerobic Infections of Wounds, and the Bacteriological and Serological Problems Arising Therefrom. Special Report Series No. 39, 1919, London. Robertson, Muriel: Brit. Med. Jour., 1918, 1, p. 583. Meyer, K. F.: Jour. Infect. Dis., 1915, 17, p. 458.

[3] Sittler: Die Wichtigsten Bakteientypen der Darmflora beim Säugling, 1909, p. 42.

[4] Chiefly by the addition or elimination of certain nutritional energy compounds.

impurity of the original cultures. Such being the case, it is readily comprehended that from the original mixture one or another of the contaminants developed greatly in excess of the remaining bacilli, as the conditions created in the cultural mediums accelerated or retarded the respective symbiotes, thus suggesting superficially that such a "dimorphism" actually existed. Still another instance of so-called anaerobic transformation is reported by Rosenthal.[5] He believed it was possible to transform the bacillus of Achalme (B. welchii B. perfringens) into the enterococcus of Thiercelin (Micrococcus ovalis), through a process of acclimatization to exposure to oxygen, until the culture became fully tolerant to the air. When the culture became fully tolerant to the air, the anaerobic bacillus lost its bacillary morphology and characteristics and assumed those of the enterococcus. Here, again, the original culture contained both the Welch bacillus and enterococcus as unrecognized symbiotes. B. welchii is now recognized as one of the anaerobic group which is commonly associated with certain putrefactive anaerobes, as Bacillus sporogenes, or members of the plectridial anaerobic group, and with Micrococcus ovalis. Cultures of the Welch bacillus of undoubted purity, however, never exhibit noteworthy variance in morphology, cultural or serologic reactions.

These examples of unrecognized symbiotism, which represent common experience in the past, are quoted as illustrative of the difficulty of obtaining and maintaining "bacteriologically pure" strains of anaerobic organisms. In defense of those investigators who have recorded these observations, which are at variance with current ideas of specific stability of bacterial types, it may be said that the plating methods used by them, so successful in separating aerobic and facultatively anaerobic organisms from one another, are wholly unreliable as a means for isolating strictly anaerobic bacilli in pure culture. The underlying reason for this marked difference in behavior between the two groups or organisms is at present not satisfactorily determined.

Bacteriologists are now in accord in recognizing that the isolation of single organisms by the Barber method[6] is the most satisfactory basis for the creation of strains of undoubted purity. The method of Barber is highly successful in the study of aerobic and facultatively anaerobic organisms. When it is applied to the anaerobic bacilli, however, the results have been disappointing. This is apparently not

[5] L'aérobisation des microbes anaérobies, 1908.
[6] Kansas Univ. Sc. Bull., 1907, 4, p. 3.

due to an inability to isolate single cells, but rather to the almost uniformly barren results of attempts to secure growths from the primary isolation.

The difficulty is explained in part at least by the unavoidable exposure of the anaerobic vegetative cells to the oxygen of the air. It is well known that the exclusion of more than minimal traces of oxygen from culture mediums is essential for the development of anaerobes, particularly in the earlier hours of growth; hence, one factor of paramount importance for single cell isolations would appear to be the exclusion of air from the material under observation. This may be accomplished either by the use of somewhat cumbersome apparatus during the process of isolation, or it may be accomplished indirectly by taking advantage of the fact that nearly all anaerobic bacilli form spores under suitable conditions; these spores do not appear to be influenced markedly by exposure to the atmosphere, although of course they fail to germinate unless air is excluded.

The first step, therefore, in an attempt to secure pure cultures of anaerobes by the Barber method would appear to be the isolation of single spores from sporulated cultures, previously heated to 80 C. for 10 minutes to kill all vegetative cells. This has been proved to be feasible, and the percentage of successful subcultures thus obtained was found to be materially in excess of that obtained when vegetative cells were isolated in place of spores. The second step which has proved to be of advantage is to place the medium containing the single spore in a vacuum oven,[7] and incubate for a few hours at body temperature[8] in a high vacuum. The combination of spore isolation, and incubation in a high vacuum, has raised the percentage of successful isolations from 1 or 2% to 35%, on the average.

The Barber pipet, as originally described, presents some difficulties of manufacture to the unskilled glass blower, particularly with reference to securing a capillary end of proper size at exactly a right angle to the main portion of the tube. A modification has been substituted which seems to fullfil all the requirements of the apparatus, and in addition to require no degree of precision in its preparation. The pipet, as finally modified, consists of a bent glass tube set in and moved by the regular Barber mechanical pipet holder. The bent end is curved at a right angle in such a manner as to present the lumen of the

[7] The De Freas vacuum oven is well adapted for this purpose.

[8] Bacilus botu'inus should be grown at 30 instead of at 38 C.

tube in the optical axis of the microscope. A small plug of modeling clay (or a small rubber stopper with a minute central hole) is used to close this end. Sterile capillary tubes of appropriate diameter and length [9] may be inserted in the clay plug (or rubber stopper) so as to move upward by manipulation of the vertical rack and pinions of the mechanical pipet holder to a point directly under the center of the lens of the microscope. The adjustment of such a capillary tube by the pipet holder in such a manner that the open end of the former is clearly visible through the microscope is a comparatively simple technical procedure, readily acquired by practice. The sterile capillary tube is adjusted so that it moves directly upward in the optical axis of the microscope; then it is lowered to a point well below the level of the stage, and a coverglass, previously sterilized, and infected with a drop of glycerine or gelatin bouillon,[10] containing spores, is suspended drop downward, on a shallow, four-sided frame moved by a mechanical stage. The bacterial spores are viewed practically as a "hanging drop" preparation in the usual manner. Any portion of the drop may be brought into the microscopic field by suitable movements of the rectilinear motion of the mechanical stage.

The process of isolating a spore is simple. The spore is located in the preparation, and the surrounding medium is searched for other spores by suitable manipulation of the mechanical stage. If the spore is well isolated, it is again brought into the center of the microscopic field, and the capillary pipet is brought into contact with the under side of the drop at precisely the spot where the spore is located. Capillarity pulls a small amount of medium, including the spore, into the pipet, which is then lowered. The capillary tube is broken off above the clay plug and below the drop of culture medium, with sterile forceps, and the contained spore is transferred to a tube of suitable medium,[11] which is placed in the vacuum oven at 37° C. When the requisite number of tubes containing spores are obtained, the vacuum is created, which draws the minute bubble of air out of each capillary tube, and the medium into it. Each spore is thus localized in a nutrient medium free from oxygen and advantageous for development.

[9] Capillary tubes are drawn out from ordinary glass tubing to the proper degree of fineness, cut into inch lengths, and sterilized in Petri dishes at 180° C. for one hour.

[10] The gelatin or glycerol is added to increase the viscosity of the medium somewhat, thus preventing too free a flow of fluid into the capillary pipet.

[11] It is essential that the medium be heated for some time at the temperature of boiling water. preferably in the Arnold sterilizer, to remove all air. It is then cooled rapidly and inoculated.

The removal of a capillary tube from the holder and the adjustment of a fresh tube in the optical axis of the microscope is the work of less than two minutes. Danger from contamination appears to be minimal, judging from the results of several hundred isolations. When the apparatus is set up, contamination from the stage is guarded against by the protection afforded by the box containing the cover glass; the pipet is long enough to project nearly an inch above the plug of clay which forms the attachment to the pipet holder. In a quiet room, bacteria practically never have become attached to the sides of the capillary pipet.

Unfortunately, the isolation of single cells or single spores does not in itself guarantee pure cultures. Many years ago Theobald Smith [12] pointed out the difficulties encountered in sterilizing milk for bacteriologic purposes, and this difficulty is increased materially in handling mediums containing finely comminuted bits of meat, or other insoluble protein. Milk is a satisfactory fluid medium for use without special arrangements of a mechanical or chemical nature to exclude oxygen, provided the cream is left intact.

It has been the practice in this laboratory to utilize the medium of Von Hibler,[13] the meat mediums of Miss Robertson,[14] and of Holman [15] and of mixtures of liver and brain for the cultivation of anaerobic bacilli. The medium is dispensed in small tubes, somewhat less than a centimeter in diameter, using a fairly deep column of nutrient material. Such mediums are kept in the autoclave for 2 hours at 20 pounds' pressure, then incubated for 2 days at 37° C., and resterilized for an hour. Experience has shown that this prolonged exposure to heat and pressure kills adventitious spores. The precaution of placing the tubes loosely in the autoclave to permit free circulation of superheated steam is conducive to the success of the operation.

The anaerobes appear to grow well in the meat or brain mediums just described, without additional precautions to exclude the air, provided inoculation is practiced immediately after heating and rapid cooling. This method, however, is wholly inadequate to secure development in peptone nutrient broth cultures.

For peptone mediums not containing meat or other protein reinforcement, some additional factor is required to induce growth. The simplest, which has stood the test of practical experience, is that of

[12] Jour. Exper. Med., 1898, 3, p. 47.
[13] Untersuchungen über die pathogenen Anaëroben, 1908.
[14] Jour. Path. & Bacteriol., 1915-1916, 20, p. 27.
[15] Jour. Bacteriol., 1919, 4, p. 149.

Hall.[16] A test tube is constricted as shown in the illustration (Fig. 1) and sufficient medium is placed therein to cover the constriction with a layer at least 3 or 4 cm. deep. A porcelain ball or a glass marble, nearly the diameter of the tube, is dropped in and the entire apparatus is sterilized in the customary manner. The presence of the ball effectually maintains a condition of anaerobiosis, of sufficient degree to permit the development of almost all anaerobic bacilli, even in sugar-free nutrient broth.[17]

A modification of this apparatus, containing somewhat more than 100 c c up to the constriction, has been found very useful in studying the metabolism of anaerobes, when considerable volumes of fluid are required for the several analyses to be made from each culture (Fig. 2).

The flasks are preferably cooled rapidly after preparation (although this is not absolutely necessary), and inoculation is readily accomplished by tilting the flask sideways until the ball rolls out of its seat to the side of the tube. A passage is thereby opened to the underlying medium, into which a drop of active culture practically invariably induces growth. The flask is righted after inoculation, and the ball again seated on the constriction, thus sealing the underlying fluid from the oxygen of the air. Of course the flasks are plugged in the ordinary manner with cotton.

The escape of gas incidental to development is readily accomplished. When the pressure of gas within the flask increases somewhat, the ball automatically lifts, the gas escapes and the ball reseats itself. It is necessary to heat and to cool the flasks slowly in order to prevent violent boiling at the constriction. If this happens, the pressure within the flask may become sufficient to expel some of the contents violently and wet the cotton plug.

These methods of isolation and of cultivation of anaerobic bacilli have been more successful than others thus far studied, but it is freely admitted that the method of isolation of single cells or single spores by the Barber method is time consuming and tedious; the results are much less constant when working with anaerobic bacilli than when isolating aerobes. A method which will combine reasonable speed with unqualified accuracy is urgently needed. The claim for recognition that the single cell method presents, however, is the possibility of obtaining results of undoubted integrity, and this factor alone far outweighs any less trustworthy means in the present state of information about the group of anaerobic bacteria.

[16] University of California Publications in Pathology, 1915, 2, p. 147.

[17] A layer of paraffin oil may be placed above the medium to restrict evaporation; it does not, however, of itself maintain anaerobiosis.